Supplemental Experimental Procedures

Generation of Cxcl12 conditional knockout mouse model

Cxcl12 specific probes were used to screen a 129Sv/Ev bacterial artificial chromosome (BAC) library. A conditional *Cxcl12*-targeting vector was constructed by retrieving the regions from BAC *Cxcl12* DNA by recombineering into PL253 and PL451 vectors. The final vector (close to 20 Kb size, with long arm of 8.1kb, short arm 4.0 kb and 7.0 kb LoxP flanked region) was linearized with *Sal1* and electroporated into TCI embryonic stem cells (129/SvEvTacfBr) by Embryonic Stem Cell Core at Nationwide Children's Hospital (Columbus, OH). The ES cell clones were selected with Neomycin/Gancyclovir and the correct recombinants were identified by Southern blot using labelled probe A resulting in a 16.8 KB wild type band and 8.2 KB band in recombination occured (Supplementary Figure S1). Selected ES clones were injected into C57BL/6 blastocysts to generate chimeric founder mice. Founder offspring were interbreed with *Actine-FLPe* and *Sox2-Cre* mice to produce conditional floxed alleles ($Cxcl12^{f}$) and heterozygous offspring lacking *LoxP*-flanked region ($Cxcl12^{-/+}$) respectively (Figure 1). Dead embryos were identified by their small size, absence of a beating heart and signs of necrosis. Genotyping was performed on tail DNA by Southern blot and/or polymerase chain reaction (PCR) using allele-specific primers described in Supplementary Figures S1 and S2. The conditional mice were back-crossed with FVB/J wild type (WT) mice for up to 10 generations to convert their mixed background to FVB/J. The $Cxcl12^{f/f}$ conditional mice were cross bred with *Fsp-cre* to generate ($Cxcl12^{f/f}$*;FSP-Cre*, abbreviated as $Cxcl12^{\Delta/\Delta}$).

Histology, Immunohistochemistry and immunofluorescence

Mouse tumors and lungs were fixed by paraformaldehyde (Affymatrix, USA) and Boine's solution (Sigma, USA) respectively, paraffin processed, cut in 4-πm sections and stained with

Hematoxylin and Eosin (H&E) using standard protocols. For immunohistochemistry (IHC) slides were processed as previously described[28] or stained using a BOND RX autostainer (Leica) as per the manufacturer's instructions using primary antibodies against Cxcl12 (1:200; Abcam, 25117), KI-67 (1:100; Biogenex, MU297-UC or Abcam, 16667), cleaved Caspase-3 (1:100; Cell Signaling, 9661), and CD31 (1:300; Santa Cruz, 1506) for 60 minutes at room temperature or overnight at 4 °C. Vectastain Elite ABC reagents (Vector Laboratories), using avidin DH: biotinylated horseradish peroxidase H complex with 3,30-diaminobenzidine (Polysciences) and Mayer's hematoxylin (Fisher Scientific), were used for detection of bound antibodies in IHC. IHC staining was scored with Allred score method[30]. The fibroblasts were identified as spindle-shaped cells with elongated nucleus present in the tumor stroma and outside the tumor nest. Immunofluorescence (IF) was performed on paraffin-embedded tissues cut into 4μM sections. The sections were deparaffinized with xylene followed by the antigen retrieval with boiling sodium-citrate buffer (pH=6) for 20 minutes (tumor samples) or 30 minutes (normal mammary glands). The sections were blocked with 5% goat serum in TBS buffer followed by staining with α-SMA (1:300; Sigma, A2547), ZO-1 (1:100; Cell Signaling, 8193), Occludin-1 (1:100; Life Technologies, 331588), VE-Cadherin (1:200; Cell Signaling, 2158), F4/80 (1:50; Bio-Red, MCA497GA), anti-goat Alaxa-Floor 568 (1:500; Life Technologies, A11057), anti-mouse Alexa-Floor 568 (1:500; Life Technologies, A10037) and anti-Rabbit Alexa-Floor 488 (1:500; Life Technologies, A21206). Sections were mounted by VECTASHIELD mounting media containing DAPI (Vector Laboratories, Inc.). Images were captured by LSM 700 confocal microscopy (Zeiss, Germany) in blinded manner. The CXCL12 expressing stromal fibroblasts in normal mammary glands were identified by pathologist based on their elongated morphology.

Western Blot Analysis

The cells were lysed with RIPA buffer (Thermo Scientific) supplemented with Protease and Phosphatase inhibitor cocktail. The protein complexes were solubilized in 4× LDS sample buffer, boiled, and subjected to SDS-PAGE using Invitrogen electrophoresis system. The proteins were transferred onto nitrocellulose membranes. The membranes were then blocked in 5% nonfat milk protein for 1 h at 37 °C and probed with primary antibody for 1 h at room temperature or at 4 °C overnight. Immuno-reactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemi-luminescence system (ECL, Amersham Biosciences).

Polymerase Chain Reaction (PCR)

The genomic DNA was isolated from mouse tail using Wizard Genomic DNA purification Kit (Promaga, A1120) as described by the manufacturer, which included optional RNase treatment before DNA precipitation. The specific primers used for genotyping are listed in Supplementary Figure S1. To specifically identify deletion of *LoxP* flanked region, we designed primers (*LoxP1*-F and *LoxP2*R) outside both the *LoxP* sites (Supplementary Figure S1). The *Cre* mediated deletion of *LoxP* flanked region generated PCR product of 523bp. The wild type allele sequence flanked by LoxP1-F and LoxP2R is 7458bp, which could not be amplified with the adopted PCR strategy and resulted in no PCR product (Supplementary Figure S1 B&E).

Quantitative real time PCR

Standard methods were used to perform real-time PCR. The reactions were normalized to *Gapdh* expression using the ΔΔCt method. *Cxcl12* primers are- forward: 5'-CAGAGCCAACGTCAAGCA-3' and reverse: 5'-AGGTACTCTTGGATCCAC-3'. *Gapdh*

primers are- forward: 5'-GCCAAACGGGTCATCATCT-3' and revers: 5'-CTAAGCAGTTGGTGGTGCAG-3'.

Estrous cycle staging

The mouse estrous cycle stages were identified according to the standard procedure (*30*). Briefly, the cytology was performed by collecting vaginal fluid in 50 µl sterile PBS and smear dried on glass slide. The slides were heat dried at 60ºC for 10 min and permeabilized with methanol. The smears were stained with 0.1% crystal violet stain and observed under light microscope.

Flow cytometry analysis

Standard methods were used to perform flow cytometry. The tumor samples were washed with 1% gentamicine (10 mg/ml, Fisher Scientific# 15710064) in DMEM complete media (10% fetal bovine serum and 1% penicillin and 1% streptomycin), followed by digestion with 2 mg/ml collagenase (Life Technologies, USA # 17104019) and 250 units of hyaluronidase (Millipore# 385931) for 1 hour at 37°C. The enzymes was neutralized by complete DMEM. The digested tumors were filtered through cell strainer (Thermo Fisher Scientific# 22-363-547) to prepare single cell suspension (SCSs). The SCSs were stained with anti-Sca1 Alexa Floor-488, anti-CD31 APC and anti-CD49b Alexa Floor-488 along with proper control IgG antibodies for 30 minutes on ice. All the antibodies were purchased from BioLegend (San Diego, CA). After staining, data was acquired by FACS Caliber using Cell Quest software (BD Biosciences) and data analysis was done using FlowJo software (Orlando, USA).

For Cxcl12 mRNA analysis in CAFs and TAMs, the SCSs were incubated with biotin labelled following antibodies- CD11b (BioLegend#101203), F4/80 (Biolegend# 123105), CD3 (Biolegend# 100243), and CD90.1 (Milteny Biotech# 130-112-871). The CD3 negative and CD90.1 positive CAFs and CD11b and F4/80 double positive TAMs were isolated by using anti-

biotin antibodies conjugated with magnetic beads and MACS milteny cell isolation setup (Milteny Biotech, USA). The CD90+/CD3- fibroblasts were allowed to attach to the bottom of culture dish for 30 minutes. Unattached cells were removed by washing the plate with fresh media three times. The mRNA was isolated and converted to cDNA by following standard protocol and using Trizol reagent (Ambion, USA# 15596026) and high capacity cDNA synthesis kit (Applied Biosystems, USA# 4368814). The Cxcl12 mRNA expression was analyzed by using Taqman gene expression analysis system (probe# Mm00445553_m1 Thermo Fisher Scientific, USA# 4331182).

Supplementary Figure S1. *Cxcl12* gene targeting and genotyping.

(A) Genomic DNA isolated from embryonic stem (ES) cell clones was digested with *EcoR1* and hybridized with *Cxcl12* probe A. (A-O are ES cell clones; +, positive control; -, negative control).

(B) Diagrammatical presentation of PCR primers location on *Cxcl12* targeted allele.

(C) List of PCR primers used to genotype mice.

(D) Genomic DNA was isolated from mouse tail and PCR was performed using specific primers to identify mouse genotype. -/- , *Cxcl12*$^{f/f}$*;Sox2-Cre*; +/f, *Cxcl12*$^{+/f}$, +/+, *Cxcl12*$^{+/+}$. f is *Cxcl12* floxed allele flanked by LoxP sites, + is Cxcl12 wild type allele.

(E) Details of PCR primer pair and resulting product size (bp; base pairs, NA; Not Available)

Supplementary Figure S2. Deletion of *Cxcl12* in fibroblasts.

(A) The pictures showing 120 days old *Cxcl12*$^{f/f}$ and *Cxcl12*$^{\Delta/\Delta}$ mice.

(B) Genomic DNA was isolated from mouse tail and genotypes were identified by PCR. M is molecular ladder.

Supplementary Figure S3. Analysis of CXCL12 expression in PyT tumors.

(A) Representative images of IHC analysis for CXCL12 expression in the tumors derived from *Cxcl12*$^{f/f}$*;PyT* (*f/f;PyT*) or *Fsp-cre;Cxcl12*$^{f/f}$*;PyT* (Δ/Δ*;PyT*) females.

(B) Quantification of CXCL12 positive macrophages per 40X magnification field of tissue sections from (A). N= 15 each group, 5 random fields from 3 different tumors. ***, p <0.001 (comparison of CXCL12 positive cells between *f/f;PyT* and Δ/Δ*;PyT*); two sample t-test.

(C) RNA hybridization analysis of PyT tumor sections with the indicated genotypes using CXCL12 and F4/80 specific probes.

(D) Quantification of CXCL12 positive macrophages per 40X magnification field of tissue sections from (C). N= 15 each group, 5 random fields from 3 different tumors. ***, p <0.001 (comparison of CXCL12 positive cells between *f/f;PyT* and Δ/Δ*;PyT*); two sample t-test.

(E). Quantitative real-time PCR analysis of CXCL12 in CD3 negative & CD90.1 positive fibroblasts and CD11b & F4/80 double positive macrophages isolated from *f/f;PyT* tumors.

S, stromal compartment; T, tumor compartment. Scale bar 20μm. *** is p<0.001, two-sample t-test.

Supplementary Figure S4. Depletion of fibroblasts derived CXCL12 inhibits mammary tumor growth.

(A) Cohorts of *f/f;PyT* or Δ/Δ*;PyT* females were evaluated for tumor growth and harvested tumors were imaged.

(B) IHC of mammary gland sections with the indicated genotypes using CC3-specific antibody (brown). Scale bar is 100μm.

(C) Quantification of Ki67 positive epithelial cells per 40x magnification field of tissue sections from (B). N= number of fields examined from total of 5 mice per genetic group. p= NS (not significant); two sample t-test.

(D) IHC of mammary gland sections with the indicated genotypes using Ki67-specific antibody (brown). Inset represents high magnification image of boxed area.Scale bar is 100μm.

(E) Quantification of Ki67 positive epithelial cells per 40x magnification field of tissue sections from (D). N= number of fields examined from total of 5 mice per genetic group. **, p <0.01; two sample t-test.

Supplementary Figure S5. Depletion of fibroblasts-specific CXCL12 inhibits mammary tumor lung metastasis.

(A) Schematic diagram of experimental design for the transgenic tumor metastasis model study. The *f/f;PyT* were euthanized at 85 days and Δ/Δ*;PyT* at 100 days to match tumor size.

(B) Tumors were harvested from (A) and weight was analyzed. Two sample t-test; p= NS (not significant).

(C) M1-M10 are images of lungs harvested from (A).

(D) H&E stained sections of lungs from (A).

(E) H&E stained sections of lungs from (D) were analyzed by ImageJ software and the metastatic nodule area (pixels) is presented as a percentage of the total surface lung area (pixels). **, p<0.01; two sample t-test. N= number of mice analyzed in each group.

Supplementary Figure S6. Effect of fibroblast-derived CXCL12 on lung metastasis in orthotopic syngeneic tumor model.

(A) Mouse mammary tumor cells (MVT1) were injected to mammary fat pad of *Cxcl12*$^{f/f}$ (*f/f*-MVT1) and *Cxcl12*$^{\Delta/\Delta}$ (*Δ/Δ*-MVT1) mice and tumor volume was analyzed every week (N=15 mice each group). p=NS (not significant); linear mixed effects model.

(B) Weight of tumors harvested from (A). p=NS (not significant); two sample t-test.

(C) H&E stained sections of lungs from (A).

(D) H&E stained sections of lungs from (C) were analyzed by ImageJ software and the metastatic nodule area (pixels) is presented as a percentage of the total surface lung area (pixels). *** is $p<0.001$, two sample t-test. N= number of mice analyzed.

Supplementary Figure S7. Effect of fibroblast-derived CXCL12 on lung metastasis in tail vein syngeneic tumor model.

(A) MVT1 cells ($1X10^6$) were injected into the tail vein of *f/f*-MVT1 and Δ/Δ-MVT1 mice (8 mice in each group). After 15 days, mice were euthanized and lungs were harvested.

(B) H&E stained sections of lungs from (A)**.**

Supplementary Figure S8: Effect of fibroblast-derived CXCL12 on CD31+ blood vessels in orthotopic syngeneic model.

(A) Representative pictures of *Cxcl12$^{f/f}$-MVT-1* and *Cxcl12$^{\Delta/\Delta}$*-MVT-1 tumor sections stained with endothelial cell-specific CD31 antibody. Scale bar, 100μm.

(B) Quantification of vascular density in tumors as determined by the number of $CD31^+$ blood vessels (from A) per 20x field.

Supplementary Figure S9: Fibroblasts derived CXCL12 regulates tumor vasculature:

(A) Effect of CAFs-derived conditioned media (CM) on MEECs angiogenesis ability as determined by tube formation assay. The MEECs were cultured with CM derived from *f/f;PyT* or *Δ/Δ;PyT* CAFs and number of MEECs mashes was calculated using ImageJ as a measure of tube formation ability.

(B) Representative images of tube formation ability of MEECs from (A).

(C) Analysis of CD31+/Sca1+ cells in *f/f;PyT* and Δ/Δ*;PyT* tumors as determined by flow cytometry.

(D) Confocal microscopy images of *f/f;PyT*, Δ/Δ*;PyT* tumors and normal mammary gland (no tumor) sections from mice injected with Fluorescent-dextran. Scale bar is 100μm.

(E) Representative images of MVT-1 cells trans-endothelial migration from Figure 5F.

(F) The pictures showing components of transwell permeability assay. The porous membrane (8μm) of top chamber was blocked by continuous layer of MEECs and supplemented with fibroblast growth media and/or fibroblasts. The bottom chamber was supplied with fibroblast growth media containing fluorescent dextran. The arrows show direction of dextran diffusion.

(G) Vascular permeability of MEECs was analyzed using transwell assay and FITC-dextran (70 KD) in the presence or absence of CAFs isolated from *f/f;PyT* and *Δ/Δ;PyT* tumors using.

Supplementary Figure S10. Clinical relevance of CXCL12 expression in breast cancer.

(A) A human tissue microarray (TMA) consisting of 257 breast cancer samples immunostained with CXCL12 specific antibody. Samples were scored by a certified pathologist blindly and association between CXCL12 levels and clinical parameters was analyzed.

(B) Example of breast cancer samples from (A) immunostained with CXCL12-specific antibody. Arrow heads point towards epithelial cells positive for CXCL12 (Scale bar 100μm).

(C & D) Kaplan-Meier Recurrence Free Survival (RFS) and Overall Survival (OS) curves of breast cancer patients expressing high or low CXCL12 in tumor cells.

Supplementary Figure S11. Fibroblast derived CXCL12 and CD31 blood vessel density predicts breast cancer patient survival.

(A & B) Kaplan-Meier Recurrence Free Survival (RFS) and Overall Survival (OS) curves of breast cancer patients from TMA having low or high CD31 blood vessel density.

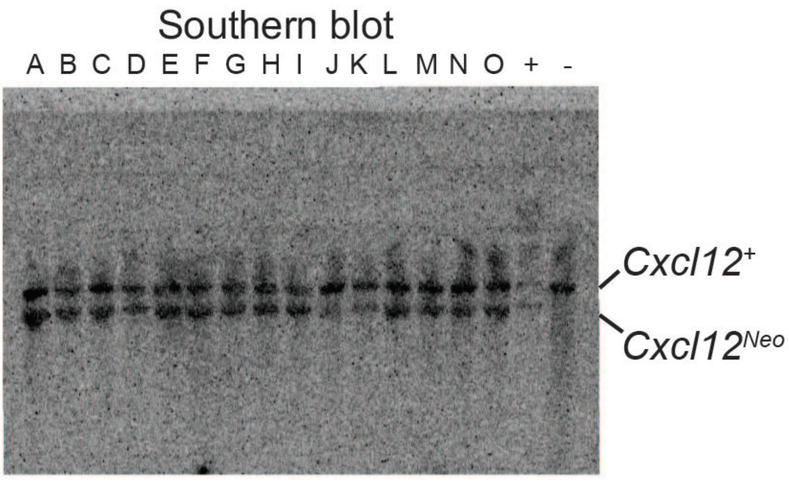
(C) Serial sections of human breast cancer sample derived from breast cancer TMA were immunostained with CXCL12 and CD31 specific antibodies. Red arrowhead points to fibroblasts and black arrowhead to CD31 blood vessels (Scale bar 100μm).

(D) Pearson's correlation analysis of stromal CXCL12 and CD31 blood vessels. Samples with blood vessels ≤ 3 are considered as low density and ≥ 4 as high density.

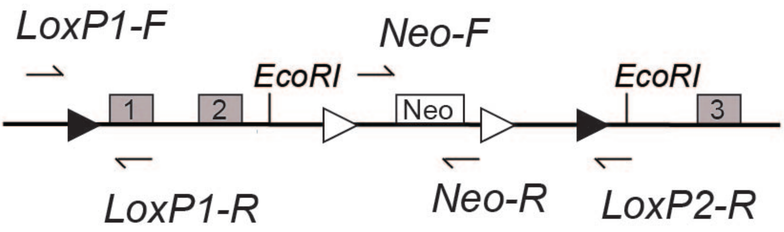
(E & F) RFS and OS curves of breast cancer patients expressing high or low CXCL12 and CD31.

Figure S1

A



B

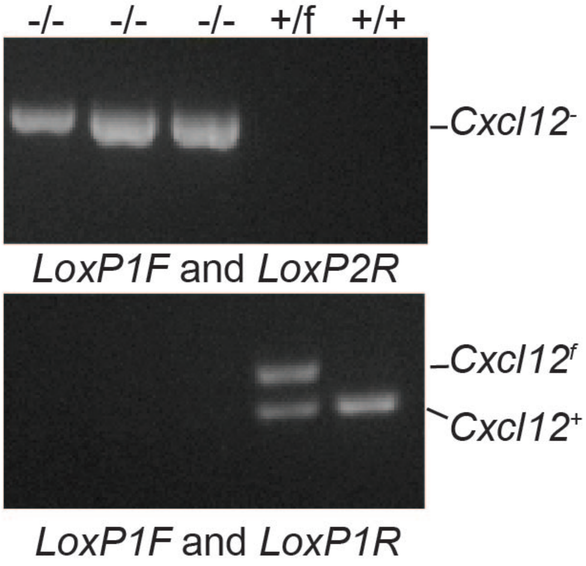


C

PCR primers sequence

Primer	Sequence (5'-3')
LoxP1-F	GCTTTGCTCTTAGCCTGCAC
LoxP1-R	CCAGATGCTCCTAGGCAAAA
LoxP2-R	CAGGACACATCTCTGCCAAG
FSP-F	TCCTGCCCTTAGGTCTCAAC
FSP-R	CCTGTTTTGCACGTTCACCG
Neo-F	ATAGCAGCTTTGCTCCTTCG
Neo-R	TGTCTGTTGTGCCCAGTCAT

D



E

PCR products (base pairs)

Primers	*Cre*	No *Cre*
LoxP1-F / LoxP1-R	261 & 227	261 & 227
LoxP1-F / LoxP2-R	523	NA
Fsp-F / Fsp-R	500	NA
Neo-F / Neo-R	NA	357

Figure S2

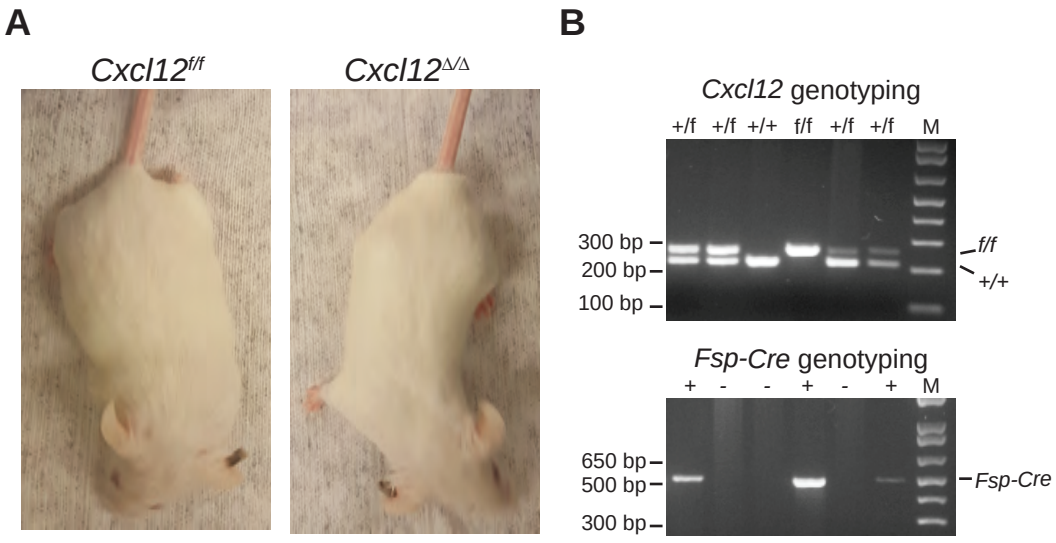
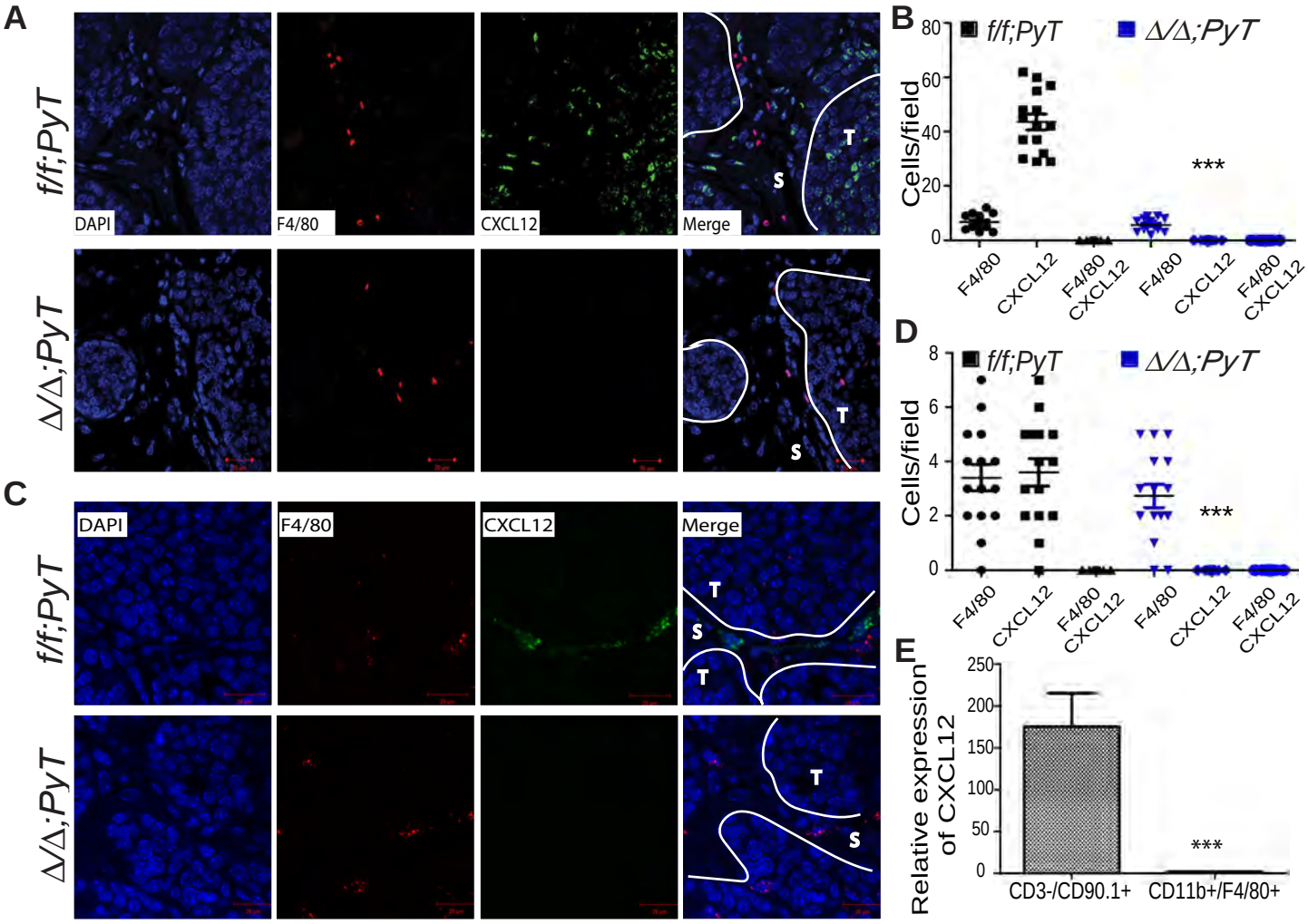

A
Cxcl12f/f
Cxcl12Δ/Δ
B
Cxcl12 genotyping
+/f +/f +/+ f/f +/f +/f M
300 bp
200 bp
100 bp
f/f
+/+
Fsp-Cre genotyping
+ - - + - + M
650 bp
500 bp
300 bp
Fsp-Cre

Figure S3


A
f/f;PyT
Δ/Δ;PyT
DAPI
F4/80
CXCL12
Merge
T
S
B
f/f;PyT
Δ/Δ;PyT
Cells/field
***
F4/80
CXCL12
F4/80 CXCL12
C
D
E
Relative expression of CXCL12
CD3-/CD90.1+
CD11b+/F4/80+

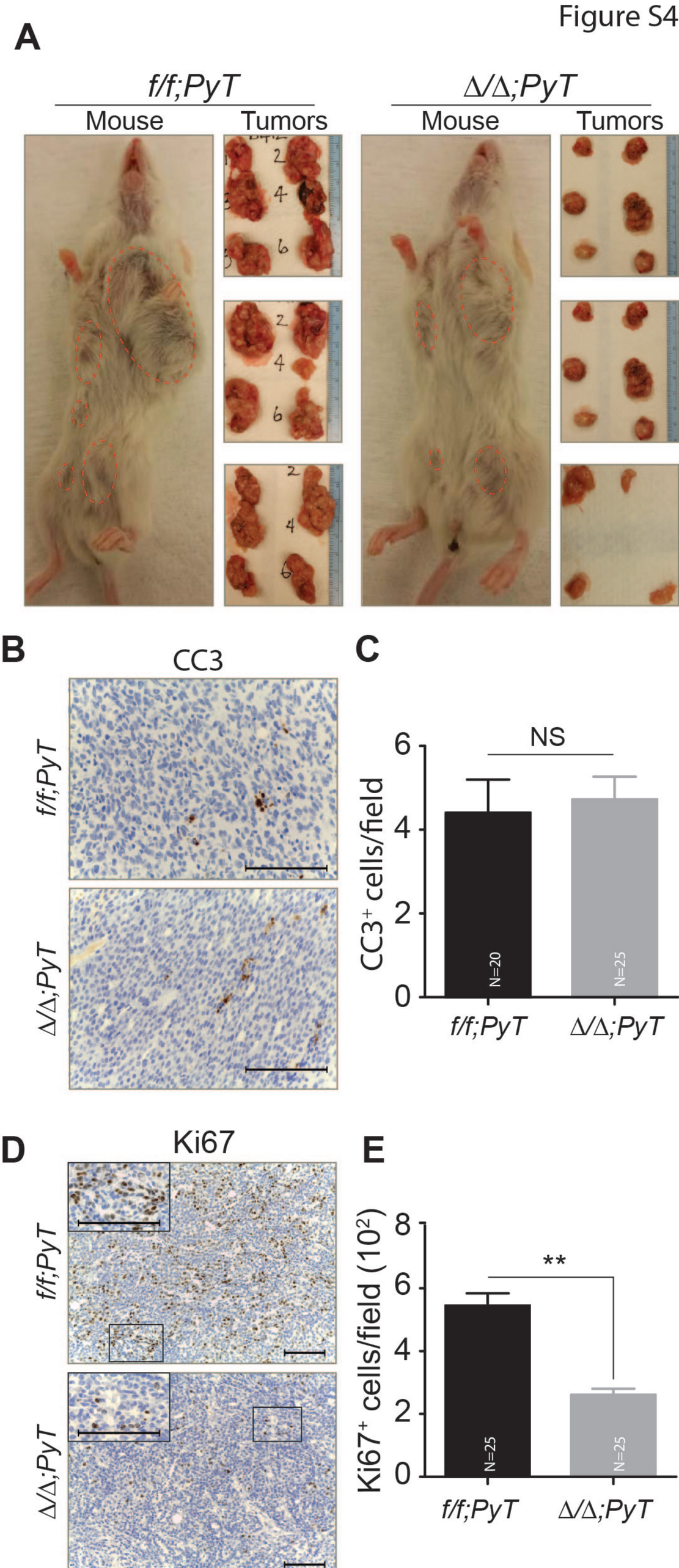
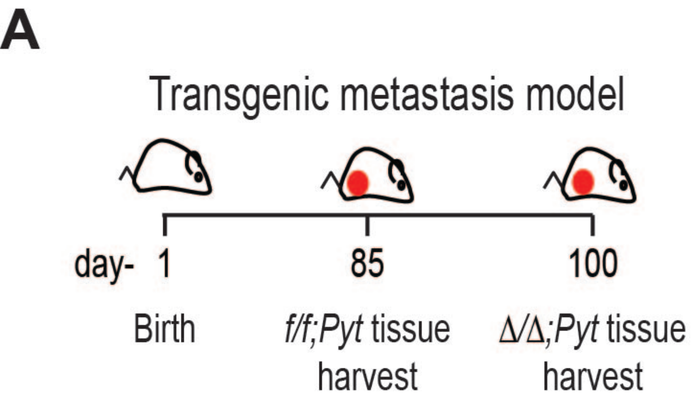
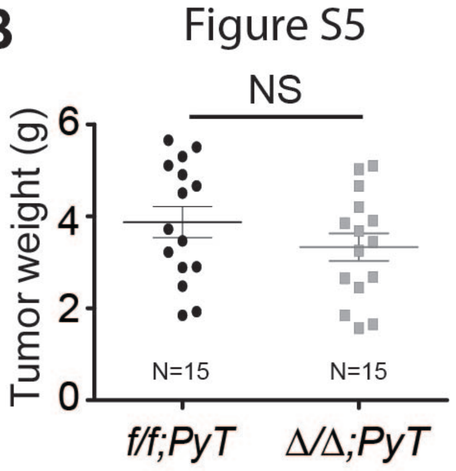
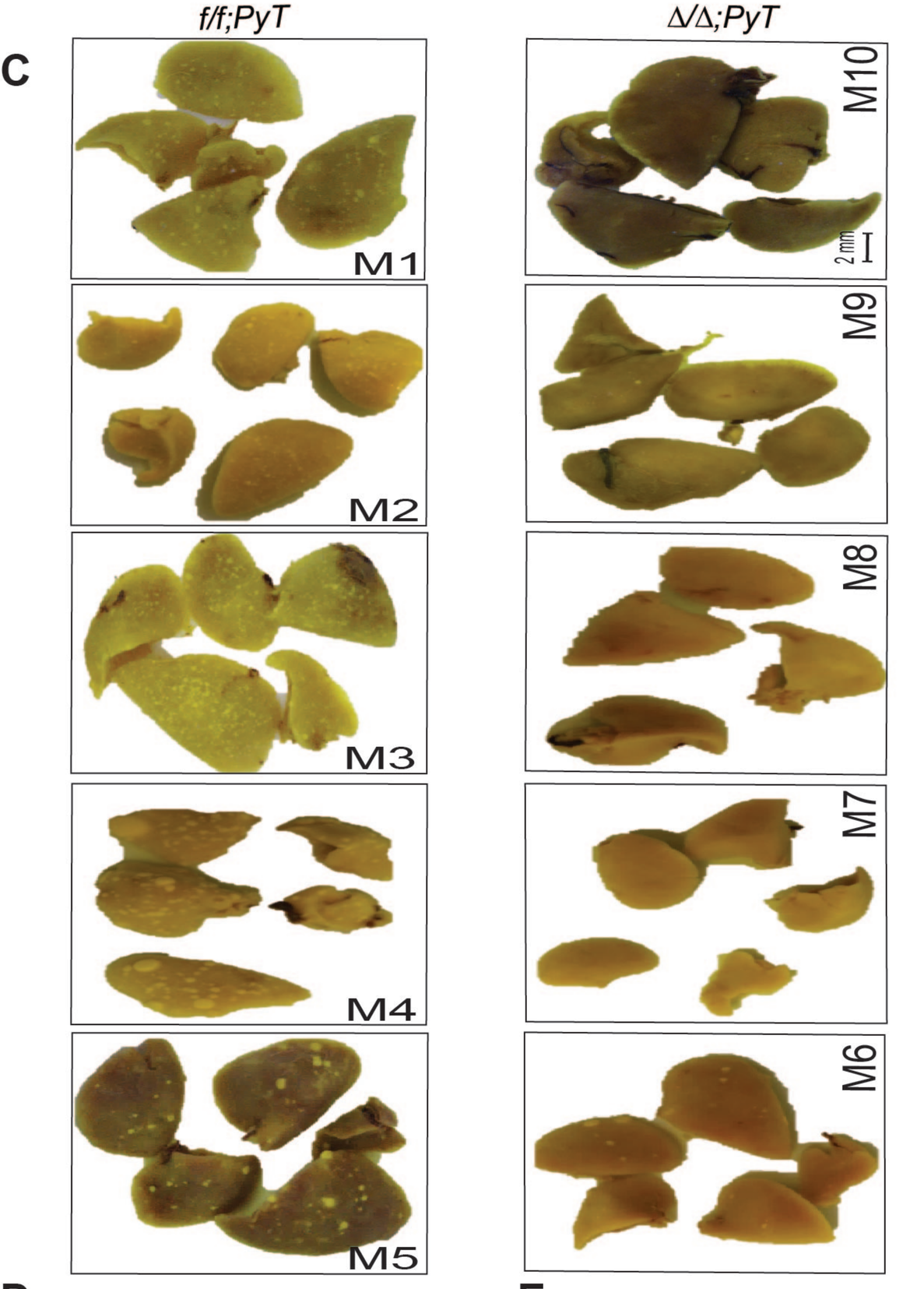
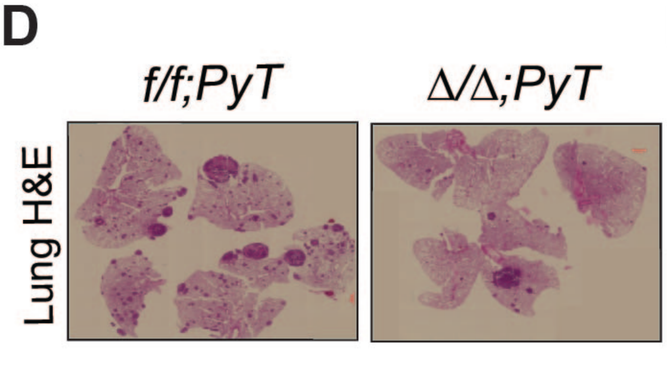

A
f/f;PyT
Mouse
Tumors
Δ/Δ;PyT
Mouse
Tumors
B
CC3
f/f;PyT
Δ/Δ;PyT
C
NS
CC3+ cells/field
6
4
2
0
N=20
N=25
f/f;PyT
Δ/Δ;PyT
D
Ki67
f/f;PyT
Δ/Δ;PyT
E
**
Ki67+ cells/field (10²)
8
6
4
2
0
N=25
N=25
f/f;PyT
Δ/Δ;PyT

Figure S5


A
Transgenic metastasis model
day- 1
85
100
Birth
f/f;Pyt tissue harvest
Δ/Δ;Pyt tissue harvest


B
NS
Tumor weight (g)
6
4
2
0
N=15
N=15
f/f;PyT
Δ/Δ;PyT


C
f/f;PyT
Δ/Δ;PyT
M1
M2
M3
M4
M5
M10
M9
M8
M7
M6
2 mm


D
f/f;PyT
Δ/Δ;PyT
Lung H&E

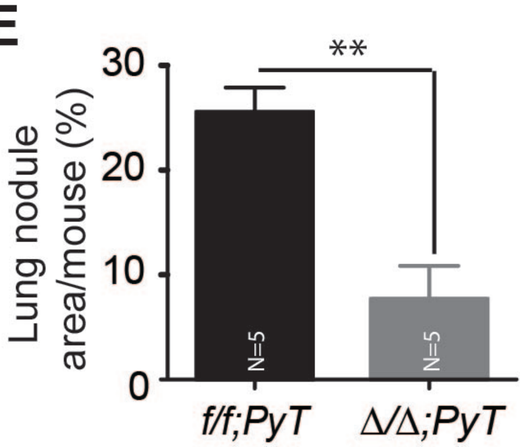

E
**
Lung nodule area/mouse (%)
30
20
10
0
N=5
N=5
f/f;PyT
Δ/Δ;PyT

Figure S6

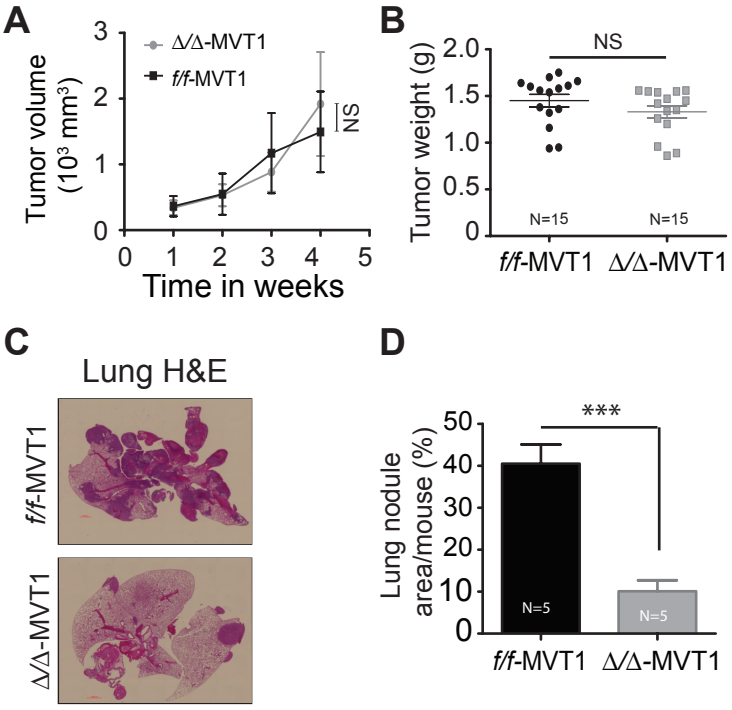
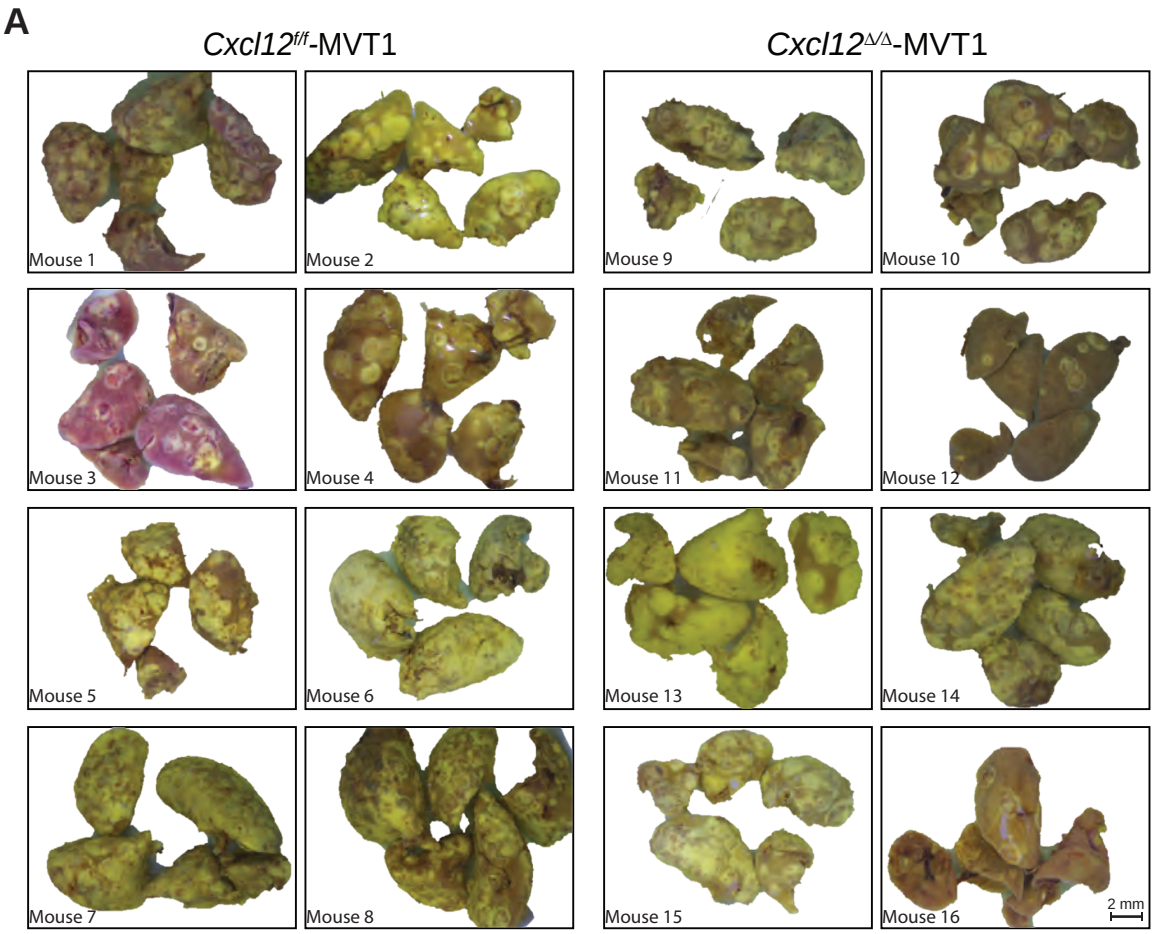

A
Δ/Δ-MVT1
f/f-MVT1
Tumor volume (10³ mm³)
NS
Time in weeks
B
NS
Tumor weight (g)
N=15
N=15
f/f-MVT1
Δ/Δ-MVT1
C
Lung H&E
f/f-MVT1
Δ/Δ-MVT1
D
***
Lung nodule area/mouse (%)
N=5
N=5
f/f-MVT1
Δ/Δ-MVT1

Figure S7


A
Cxcl12f/f-MVT1
Cxcl12Δ/Δ-MVT1
Mouse 1
Mouse 2
Mouse 9
Mouse 10
Mouse 3
Mouse 4
Mouse 11
Mouse 12
Mouse 5
Mouse 6
Mouse 13
Mouse 14
Mouse 7
Mouse 8
Mouse 15
Mouse 16
2 mm

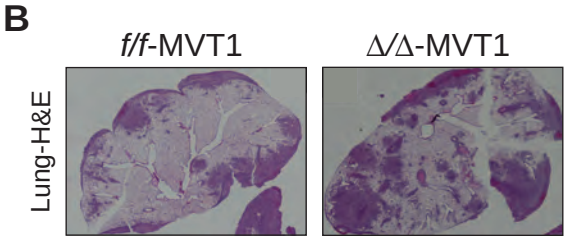

B
f/f-MVT1
Δ/Δ-MVT1
Lung-H&E

Figure S8

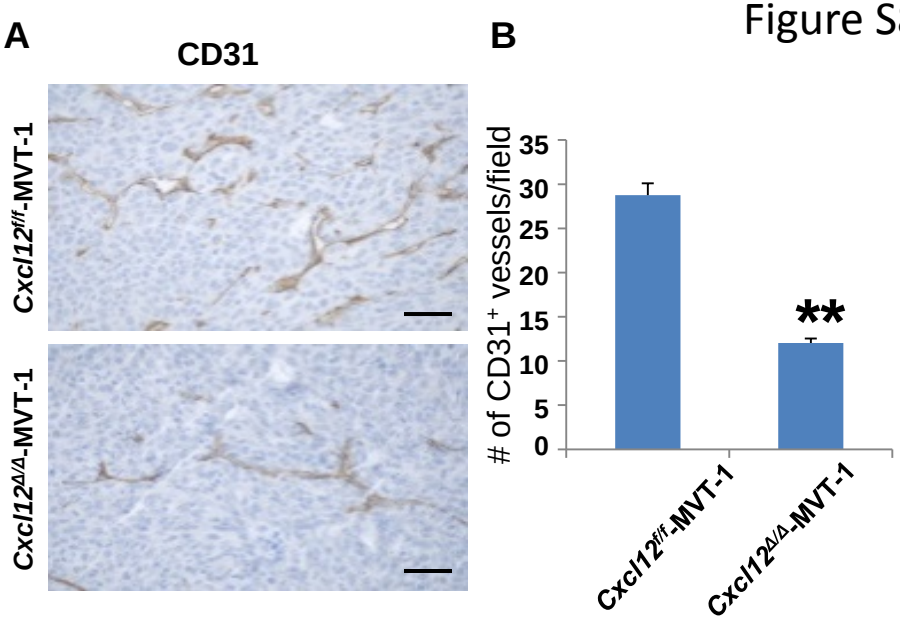

A
CD31
Cxcl12f/f-MVT-1
Cxcl12Δ/Δ-MVT-1
B
# of CD31+ vessels/field
35
30
25
20
15
10
5
0
**
Cxcl12f/f-MVT-1
Cxcl12Δ/Δ-MVT-1

Figure S9

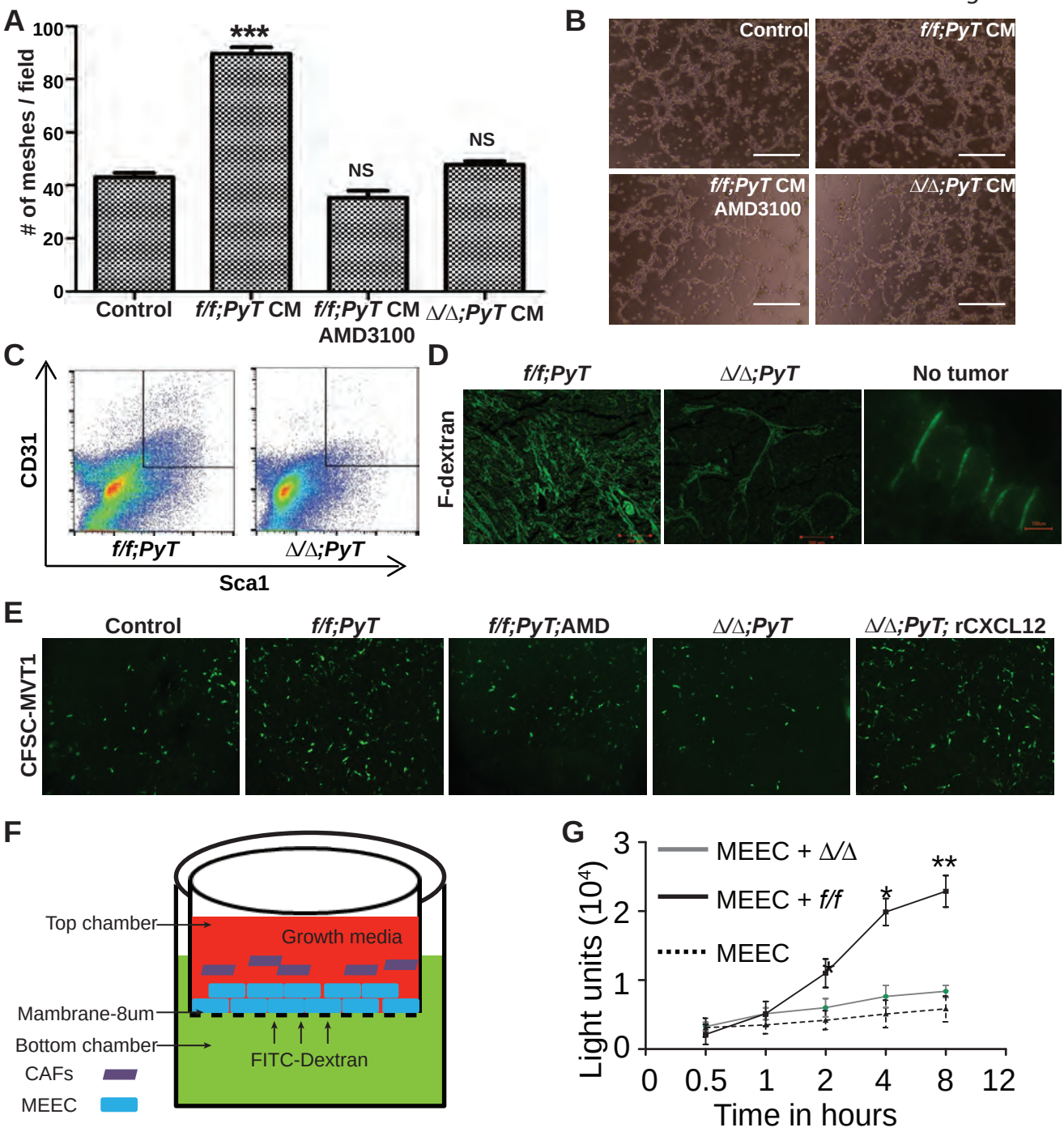

A
# of meshes / field
100
80
60
40
20
0
***
NS
NS
Control
f/f;PyT CM
f/f;PyT CM AMD3100
Δ/Δ;PyT CM
B
Control
f/f;PyT CM
f/f;PyT CM AMD3100
Δ/Δ;PyT CM
C
CD31
f/f;PyT
Δ/Δ;PyT
Sca1
D
f/f;PyT
Δ/Δ;PyT
No tumor
F-dextran
E
Control
f/f;PyT
f/f;PyT;AMD
Δ/Δ;PyT
Δ/Δ;PyT; rCXCL12
CFSC-MVT1
F
Top chamber
Growth media
Mambrane-8um
Bottom chamber
FITC-Dextran
CAFs
MEEC
G
MEEC + Δ/Δ
MEEC + f/f
MEEC
Light units (10⁴)
3
2
1
0
*
**
0
0.5
1
2
4
8
12
Time in hours

Figure S10

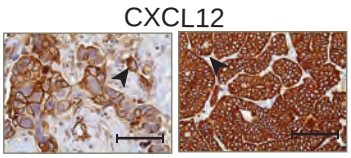
A

Associaation of stromal CXCL12 levels with clinical features in breast cancer patients

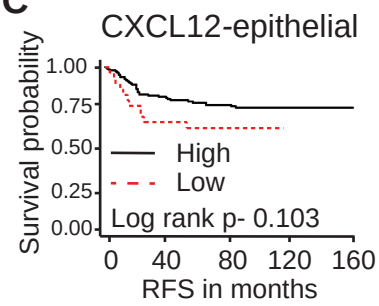
	HR	95% CI
Association of CXCL12 with high grade breast cancer		
Grade III versus grade I and II	2.220 [a]	1.07-4.61
Association of CXCL12 with high stage breast cancer		
Stage II-IV versus stage I	2.406 [b]	1.18-4.90
Association of CXCL12 with triple negative breast cancer		
Triple negative breast cancer versus others	0.349	0.04-2.73
Association of CXCL12 with ER positive breast cancer		
ER positive versus ER negative	0.423	0.17-1.05
Association of CXCL12 with PR positive breast cancer		
PR positive versus PR negative	0.560	0.26-1.20
Association of CXCL12 with Her2 positive breast cancer		
Her2 positive versus Her2 negative	0.869	0.41-1.85

Logistic regression analysis used to calculate hazards ratio (HR).
Pearson chi-square test used to determine statistical significane.
[a] and [b] significant, p=0.032 and 0.016 respectively. CI, confidence interval.
ER, estrogen receptor; PR, progesteron receptor; Her2, human epidermal growth fector receptor 2.

B



C



D

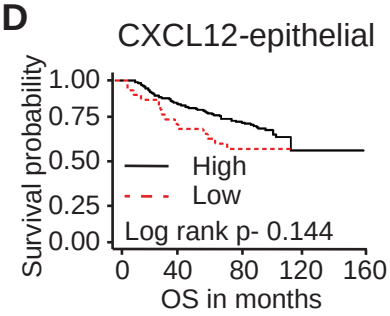
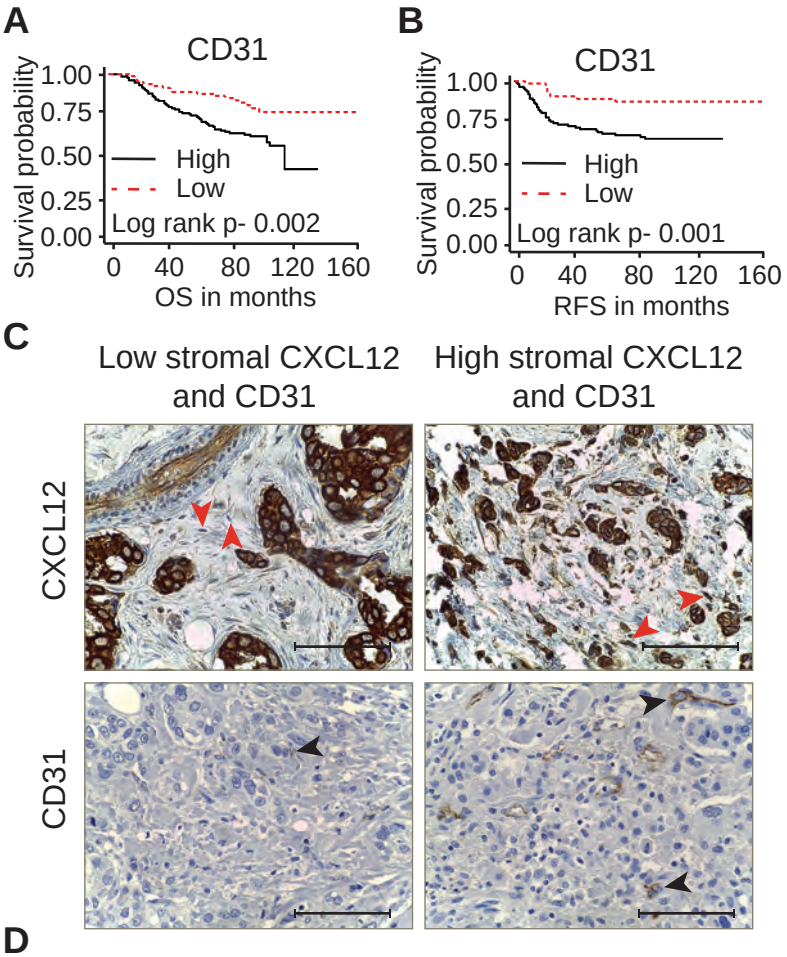


Figure S11



D

Correlation of stromal CXCL12 expression and CD31 blood vessel density

CD31 (%)	CXCL12 (%)		Total	Pearson correlation	Chi-square p value
	Low	High			
Low	20 (23)	67 (77)	87 (100)	0.125	0.020
High	16 (11.4)	124 (88.6)	140 (100)